DMD Fast Forward. Published on April 8, 2005 as doi:10.1124/dmd.104.003335

DMD #3335

Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation

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Running title: Variable Expression of MRP2 in human placenta

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Number of pages: 40

Number of tables: 3

Number of figures: 6

Number of words in the abstract: 250 words

Number of words in the introduction: 442 words

Number of words in the Discussion: 1.689 words

ABSTRACT

MRP2 (ABCC2) is an ABC type membrane protein involved in transport of conjugates of various drugs and endogenous compounds. MRP2 has been localized to the apical membrane of syncytiotrophoblasts and is assumed to be involved in diaplacental transfer of the above substances. It has been shown that both genetic and environmental factors can influence *MRP2* expression. We therefore investigated whether gestational age, cellular differentiation and genetic polymorphisms influence expression and localization of MRP2 in 58 human placenta samples. We detected a significant increase of transporter-mRNA with gestational age by quantitative Real-time PCR (MRP2 mRNA/18S rRNA-ratio x 1000 ± SD; 0.43 ± 0.13 in early preterms vs. 1.18 ± 0.44 in late preterms vs. 2.1 ± 0.63 in terms; $p < 0.05$). MRP2 protein followed the mRNA amount as shown by Western blotting (mean relative band intensity ± SD; 0.56 ± 0.1 vs. 0.7 ± 0.18 vs. 0.92 ± 0.19; early preterms vs. terms $p < 0.05$). In cultured cytotrophoblasts *MRP2* expression increased with differentiation to syncytiotrophoblasts with a peak on day 2 (MRP2 mRNA/18S rRNA-ratio x 1000 ± SD; 0.06 ± 0.01 vs. 0.88 ± 0.27 vs. 0.24 ± 0.02 on days 0, 2 and 4). Moreover, we studied the effect of single nucleotide polymorphisms (C-24T; G1249A and C3972T) in the *MRP2* gene on placental expression. One of these polymorphisms (G1249A) resulted in a significantly reduced expression of MRP2-mRNA in preterms. In summary, the expression of MRP2 in human placenta is influenced by gestational age, cellular differentiation and genetic factors.

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INTRODUCTION

Human placenta is the feto-maternal interface during pregnancy and functions as the main barrier between maternal and fetal circulation. The exchange of compounds between mother and child is facilitated by a single layered syncytium, the syncytiotrophoblast. These polarized cells are attached to the outer layer of the terminal villi consisting of fetal vessels and the villous stroma. During pregnancy the functional multinuclear syncytiotrophoblast is formed from fusion of cytotrophoblasts. The latter cells loose their proliferative activity during the maturation process (Kingdom *et al.*, 2000). Therefore, the fraction of cytotrophoblasts undergoing differentiation decreases with increasing gestational age. In vitro experiments using isolated cytotrophoblasts in cell culture showed spontaneous differentiation into syncytiotrophoblasts (Kliman *et al.*, 1986).

The ATP-binding cassette (ABC) superfamily of membrane transporters is a family of proteins known to be involved in the organization of functional barriers in human organism. The unidirectional ATP-dependent transport of substances of physiological and pharmacological relevance contributes to this function. The protective function of placenta and especially of the syncytiotrophoblasts against xenobiotics affecting fetal development is based at least in part on these transport proteins since they prohibit maternofetal transfer of potentially toxic compounds (Young *et al.*, 2003).

Different members of the ABC-superfamily are expressed in human placenta. These include proteins, as the *MDR1* (ABCB1) gene product P-glycoprotein (P-gp), the breast cancer resistance protein (BCRP, ABCG2), and several members of the MRP (ABCC) subfamily, such as MRP5 and MRP2 (ABCC2) (MacFarland *et al.*, 1994;Mylona *et al.*, 1996;Nakamura *et al.*, 1997;St Pierre *et al.*, 2000;Maliepaard *et al.*, 2001;Pascolo *et al.*, 2003;Meyer zu Schwabedissen *et al.*, 2005) The latter transporter had initially been designated as canalicular multispecific organic anion transporter (cMOAT). MRP2 is involved in the hepatobiliary excretion of conjugated bilirubin and conjugated drugs which are metabolites of the phase II-

biotransformation. Moreover, MRP2 facilitates transport of anticancer agents, including cisplatin, vinblastin, and camptothecin derivatives (Borst *et al.*, 2000)

In previous studies it has been shown that the Dubin-Johnson-syndrome, an autosomal-recessive disorder is linked to the absence of MRP2 in human liver resulting in symptomatic conjugated hyperbilirubinaemia. Besides the known mutations in Dubin-Johnson syndrome, a number of single nucleotide polymorphisms have been recently reported, which do not cause the Dubin-Johnson phenotype (Gerk and Vore, 2002).

In summary, MRP2 can function as an important protective mechanism in human placenta. Whether environmental and / or genetic factors affect *MRP2* expression in this tissue is unknown. We therefore investigated the influence of gestational age, genetic polymorphisms and cellular differentiation on the expression and localization of MRP2 in placenta. In this paper we demonstrate a significant reduced expression in preterm placenta, increased expression during in vitro differentiation and an influence of the G1249A polymorphism.

METHODS:

Materials

Chorionic villous tissues were obtained from women undergoing caesarian sections and normal birth. A total of 58 samples from preterm (*n = 26*) and term placentas *(n = 32*) were included in the present study. Written informed consent was obtained in each case. The study was approved by the local ethical committee. Following the WHO (World Health Organization) definition pregnancies with duration less than 37 weeks of gestation were defined as preterm. The group of preterms was divided in early (before 32 weeks gestation) (*n = 10*) and late preterm (32 to 37 weeks gestation) (*n = 16*). Demographic data are summarized in table 1. Samples for isolation of cytotrophoblasts were taken from term placentas of normal deliveries (*n = 4*).

Cytotrophoblast culture

Cytotrophoblasts were isolated as described by Kliman et al. 1986 (Kliman *et al.*, 1986). After mechanical and enzymatic dissection of placental tissue of different term placentas (*n=4*) cytotrophoblasts were separated using density gradient centrifugation at 1 500 x g for 45 min on discontinuous Percoll-gradient (20-70%). Cells between the 40% and 50%-Percoll bands were collected and plated onto 35 mm² culture dishes at a density of 5 x 10^6 cells/dish. The cells were grown in M199 medium, supplemented with 10% FCS and 100 units/mL penicillin/streptomycin and 5 ng/mL EGF, in 5% CO_2 humidified atmosphere at 37°C. The cells were maintained in culture for five days. Every day one dish was harvested; the cells were scraped off the plates and collected by centrifugation at 800 x g at 4° for 5 min.

Control of the purity of the isolated cytotrophoblasts

The purity of the isolated cells was assessed using immunofluorescent staining of the cultured cells with an anti-cytokeratine 18 (Sigma-Aldrich, Taufkirchen, Germany) and an anti-

vimentin antibody. Cells were cultured on cover slips for 3 days. After rinsing, the cells were fixed 15 min in 4%-paraformaldehyde, washed three times with PBS (pH 7.4) and permeabilized with 0.1%-TritonX-100. Cells were washed then several times with PBS and incubated in 5%-FCS, followed by incubation over night at 4° with the primary antibodies (anti-cytokeratine diluted 1:800 or anti-vimentin diluted 1:200). After several washing steps with PBS the cells were incubated 1.5 h with the fluorescent labeled anti-mouse antibody (Alexis Biochemicals, Gruneberg, Germany), diluted 1:200. After that the labeled cells were mounted on slides using DAKO anti-fading mounting medium (DAKO, Hamburg, Germany). Staining was detected using confocal laser scanning microscopy.

Real-Time Quantitative RT-PCR:

RNeasy Mini extraction kit (Qiagen, Frankfurt, Germany) was used for RNA extraction from 32 term and 26 preterm placentas and cytotrophoblasts of four different preparations. In brief, villous material was separated and frozen in liquid nitrogen. The tissue was mechanically homogenized using a microdismembranator (1 min, 2500 rpm). Subsequently 60 mg of the tissue powder were incubated with guanidinium thiocyanate containing buffer and centrifuged through a membrane system. Binding at a silica gel membrane was performed according to the manufacturer's instructions. Finally the integrity of the RNA was controlled by ethidium bromide staining in a formaldehyde-containing 1% agarose gel.

The isolated and purified polyA-RNA was reverse transcribed using Random Hexamere primer and the TaqMan reverse transcription (RT) kit (Applied Biosystems, Foster City, CA). Quantitative Real-time PCR was used for establishing the mRNA amount. A pre-developed primer and probe mix was used for detection of 18S ribosomal RNA (Applied Biosystems, Foster City, CA). The MRP2 primer sequences were designed intron-spanning based on the cDNA sequence published under Gene Bank accession number XM_083829. In detail primer sequences for quantification of MRP2 were 5´-CTGGGAACATGATTAGGAAGC-3´ and 5´-

GAGGATTTCCCAGAGCCGAC-3´ and the fluorescent labelled probe sequence 5´-(FAM)CAFTCCGAGATGTGAACCTGGACAT-XTp. Calibration curves for MRP2-mRNA and 18S rRNA quantifications were prepared by several dilutions of cDNA-constructs ligated to pGEM®-T-Easy (Promega GmbH, Mannheim, Germany) transformed to E. coli KL-10-Gold (Stratagene, La Jolla, CA), with amounts ranging from 1×10^7 to 1×10^1 copies for MRP2 and 1×10^9 to 1×10^3 for 18S.

The RT-PCRs were set up in a reaction volume of 50 µl containing 15 ng or 0.15 ng of cDNA for amplification of the MRP2 mRNA or 18S rRNA, respectively. Amplification of the PCR products was performed using the ABI Prism 7700 Sequence detector, a Real-time PCR cycler (Applied Biosystems, Foster City, CA) and the Universal PCR mastermix supplied by Applied Biosystems. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as threshold cycle (CT). The CT value of each sample was compared to the CT values of the standard series, which consisted of the cloned PCR fragment resulting in a quantification of copy numbers mRNA. The fragment inserted in a common used vector was sequenced before using as standard showing no differences to the published cDNA sequence (Gene Bank: Accession number XM_083829) (data not shown).

Immunoblot analysis of crude membrane fractions of term and preterm placentas

Crude membrane fractions were isolated from 29 placental homogenates using a subset of 17 term and 12 preterm placentas. Frozen tissue was homogenized using a Braun-Elvejhem homogenizer in homogenization buffer (10 mmol/L Tris/HCl, 250 mmol/L Sucrose, and 1 mmol/L EDTA) supplemented with protease-inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 0.3 µmol/L aprotinin and 0.1 µmol peptastin. After gradual centrifugations at 9 000 x g and 100.000 x g the crude membrane fraction of placental tissue was resuspended in 50 µl 5 mmol/L Tris/HCl (pH 7.4). Aliquots were denatured in SDS-PAGE-sample-buffer at

37°C for 30 min. After incubation the membrane proteins were separated by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) and electrotransferred onto nitrocellulose using a tank blotting system (BioRad, München, Germany). Membrane preparation from human liver was used as positive control. For detection of MRP2 reactive bands the primary anti-MRP2 antibody M_2 I-4 (Alexis Biochemicals, Gruneberg, Germany) and the secondary horse-radish peroxidase-conjugated goat anti-mouse IgG antibody (BioRad, München, Germany) were used. The antibodies were diluted in Tris-buffered saline containing 0.05 % Tween 20 and 1% bovine serum albumin to a final concentration of 1:1000 for the MRP2 and 1:2000 for the secondary antibody. The immobilized antibodies were stained using an enhanced chemiluminescent (ECL) system (Amersham Bioscience, Freiburg, Germany) and exposed to X-Ray films. After digitalization band intensities were analyzed using the KODAC-Image Quant Software. The crude membrane fraction of a term placenta was used for relative quantification. The relative intensity of the samples to this internal standard represents the amount of protein.

Preparation of apical and basal membrane fractions:

Apical and basal membrane fractions were isolated from two term and two late preterm placentas. First placental cotelydones were washed several times with ice cold PBS, after that 20 g of the tissue were minced and washed again. Tissue was homogenized using a Braun-Elvejhem homogenizer (20 strokes, 1000 rpm) in incubation buffer (250 mmol/L sucrose and 10 mmol/l Tris/HCl and 1mM EDTA; pH 7.4) supplemented with protease inhibitors (0.1 mmol/L phenylmethylsulfonyl fluoride, 0.3 µmol/L aprotinin and 0.1 µmol peptastin). After that the homogenate was incubated 1 h on ice under continuously stirring. After centrifugation at 9.000 x g the supernatant was centrifugated at 100.000 x g. The pellets containing the membranes were resuspended in incubation buffer and homogenized with a loose-fitting Dounce B homogenizer. For separation of basal and apical membranes, $MgCl_2$

was added to a final concentration of 10 mmol/L and centrifugated at 2.000 x g after a 10-minute incubation on ice. The separated membranes - apical fraction (supernatant) and the basal fraction (pellet) - were resuspended in incubation buffer and homogenized with a tight-fitting Dounce B (30 strokes). After centrifugation at 100.000 x g the membranes were homogenized using the tight-fitting Dounce B and centrifugated at 100.000 x g. After that the pellets of both membrane fractions were resuspended in 1 ml homogenization buffer and frozen and stored in liquid nitrogen.

Laser scanning confocal microscopy:

Human chorionic villi of mature and premature placenta (41^{st} week of gestation and 26^{th}, respectively) were fixed in formalin after delivery and embedded in paraffin. Sections of 2 µm thickness were prepared on Superfrost Plus® slides and dried over night at 60°C. The paraffin-embedded sections were deparaffined in two changes of xylene substitute for 10 minutes each. After that the slides were incubated in ethanol of declining concentrations from 100% to 50% for 5min and rinsed in distilled water two times. Heat induced epitope retrieval was performed by boiling the tissue sections in citrate buffer (10mM, pH 6.0) for 15 minutes. After several washing steps in cold PBS (pH 7.4) the sections were blocked in 2% BSA. Sections were incubated with primary anti-MRP2 antibody M_2 III-6 (Alexis Biochemicals, Gruneberg, Germany) over night at room temperature in a humidified atmosphere. After several washing steps with PBS, the sections were incubated with a fluorescent-labelled secondary antibody against mouse IgG 2α (Alexis Biochemicals, Gruneberg, Germany). After mounting in anti-fading mounting medium (DAKO, Hamburg, Germany), the fluorescence was detected by laser scanning confocal microscopy. As control for background signals, sections were incubated with the secondary antibody only and scanned under the same conditions.

RFL-Polymorphism:

For studying the influence of single nucleotide polymorphisms (SNPs) on MRP2-mRNA expression 58 samples of human placentas were screened for known polymorphisms. After mechanical tissue homogenization genomic DNA (gDNA) was extracted using Qiagen Tissue Kit (Qiagen, Frankfurt, Germany) as described by the manufacturer. 100 ng of the gDNA were amplified using primers (Tab.2) flanking the regions of previously described SNPs (Ito *et al.*, 2001). These primers were used for amplification of a 301 bp-fragment in the 5´-flanking region containing the C-24T variant, of a 260 bp fragment in exon 10 containing the G1249A variant, and of a 184 bp-fragment in exon 28 containing the 3972 polymorphism. PCR was carried out in a volume of 25 µl containing 200 nM of each primer 10 x PCR Buffer, 2.5 mM $MgCl_2$, 1 mM of dNTP-Mix and 1.25-2.5 U TaqPolymerase. After amplification the PCR products were digested using sequence depending restriction endonucleases. Restriction products were identified after electrophoretic separation in a 2% agarose gel and staining by ethidium bromide.

Statistical analysis

The amounts of mRNA and protein were compared using the Student's t-test for two independent groups. $P < 0.05$ was considered as statistically significant. In addition, the statistical difference was tested using the Kruskal-Wallis-test of ranking, where indicated.

RESULTS:

MRP2 mRNA expression in total tissue of human placenta:

In order to study the influence of gestation on the expression of *MRP2* in human placenta we compared the MRP2 mRNA level in 32 term and 26 preterm placentas. The group of preterm placentas was divided in early (*n=10*) and late *(n=16*) preterms. The early preterm period was determined to 32 weeks of gestation, as this gestational age is presumed to be clinical significant in preterm mortality.

The isolated and transcribed RNA was amplified by quantitative real time-PCR. The quantification of MRP2 mRNA and 18S rRNA amount was performed using cloned PCR fragments. The measured number of MRP2 mRNA copies was normalized to those of 18S rRNA. The normalized MRP2 mRNA amount in term placentas was significantly higher than that of the early and late preterms (FIG.1). Term placentas showed an about 1.8-fold higher mean expression than late preterm placentas and an about 4.4-fold higher MRP2 expression than early preterms (mean MRP2 mRNA/18S rRNA- ratio ± SD 2.10 ± 0.24 vs. 1.18 ± 0.35 vs. 0.48 ± 0.17). We did not observe an influence of the gender of the fetus on the mRNA amount neither in the group of preterm nor of term placentas (data not shown).

Protein Expression of MRP2 in human term and preterm placenta:

MRP2 protein was detected by immunoblot analysis in 13 term and 16 preterm placentas. For analysis crude membrane fractions were prepared, separated by SDS-PAGE and electrotransferred to nitrocellulose filters. Immunoblot analysis was performed using the monoclonal anti-MRP2 antibody (M_2 I-4) yielded a double band at approximately 180kDa (FIG.2) These bands were detectable in every placental sample. As positive control human liver was used. For quantification an internal standard consisting of a term placenta crude membrane extract was loaded on every analytical gel, band intensity of the major band of other placental samples was related to this. The relative band intensity of MRP2 protein was

significantly reduced in early preterm placentas (mean relative band intensity ± SD 0.57 ± 0.1) compared to that of term placentas (mean relative band intensity ± SD 0.92 ± 0.18, Students t-test $p<0.05$). The comparison of term and late preterm placentas showed no statistical significant difference (FIG.2D).

For determination of the localization of MRP2 immunoblot analysis was performed using membrane preparations enriched in apical or basal fraction of two term and two preterm placentas. In agreement with the data shown by M.V. St-Pierre (St Pierre *et al.*, 2000) the abundant protein expression of MRP2 was found in the apical membrane fraction (FIG.3A).

Immunofluorescent staining of MRP2 in human placenta:

To study whether the effect of lower protein expression is accompanied by a different localization of the MRP2 protein in human preterm placenta, we performed immunofluorescent staining of paraffin-embedded placenta sections. As shown in figure 3 B and 3 C the immunofluorescent staining was less pronounced in preterm placentas. Localization of MRP2 in the placental villi surrounding syncytiotrophoblasts was not changed by gestational age.

MRP2 mRNA expression in cultured cytotrophoblasts:

For determination of the influence of cellular differentiation of cytotrophoblasts on the expression of *MRP2*, the mRNA amount was determined in cultured cytotrophoblasts and normalized to the amount of 18S rRNA. MRP2 mRNA was detected in all cytotrophoblast samples. Spontaneous cellular differentiation to syncytiotrophoblasts was observed by light microscopy after the first day (FIG. 4C and D). Purity of the isolate was controlled by staining with an anti-cytokeratine 18 antibody (FIG. 4A) and staining with an anti-vimentin antibody (FIG. 4B). Moreover, the maturation of these cells was determined by the biochemical marker β-hCG, a hormone, which is secreted by the multinuclear syncytium. As shown in figure 4E, the cells secreted increasing amounts of this hormone during culture. With maturation *MRP2*

mRNA amount increased and decreased (FIG. 5) independently of the respective time course of the β-hCG level in supernatant. The peak of MRP2 mRNA at the second day of culture (MRP2 mRNA/18S rRNA-ratio x 1000 ± SD; 0.88 ± 0.27) was 15.2 fold higher than that of uncultured cytotrophoblasts (MRP2 mRNA/18S rRNA-ratio x 1000 of 0.06 ± 0.01; Student's t-test $p < 0.05$) and decreased from day 2 to day 5 (FIG. 5).

MRP2 protein amount in cultured cytotrophoblasts:

As cultured cytotrophoblasts showed an increase in *MRP2* mRNA amount during differentiation, we studied if this induction is accompanied by a change in the amount of MRP2 protein. Syncytiotrophoblasts were harvested from culture after 72 h and 144 h. MRP2 protein amount of maturated cells was compared with cytotrophoblasts of the same individual by immunoblot analysis. MRP2 expression is increased in syncytiotrophoblasts compared to the cytotrophoblasts, the progenitor cells, not showing a subsequent decrease parallel to the reduced amount of mRNA (insert FIG. 5).

MRP2 Genotyping:

Three of the five SNPs previously reported in a Japanese population were found in our placental samples of Caucasian individuals. 21 placentas were heterozygous for the C to T transition 24 bases upstream from the initial codon (TAB. 3). Moreover, 5 homozygotes and 16 heterozygotes of the missense mutation G1249A (exon 10) were identified. This polymorphism causes a substitution of $valin_{417}$ by isoleucin. Interestingly, the frequency of a homozygote G 1249 carriers with 0.8 is higher in preterm placentas than in term placentas (identified frequency 0.5) (TAB. 3). The third SNP found in the examined samples was C3972T (exon 28). 9 homozygotes and 27 heterozygotes have been identified (TAB. 3). This variation at codon 1324, however, causes no missense as both triplets encode isoleucin.

DMD Fast Forward. Published on April 8, 2005 as DOI: 10.1124/dmd.104.003335
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Genotype of position -24 and MRP2 mRNA and protein amount

The amount of MRP2 mRNA expression established as described above and normalized to 18S rRNA showed no difference concerning -24C>T regardless of gestational age of the placenta (MRP2/18S-ratio x 1000 ± SD; terms: -24 CC 2.11 ± 0.34 (*n = 20*) and -24 CT 2.12 ± 0.35 *(n = 12)*; Kruskal-Wallis-test: χ^2=0.133; df=1; p=0.715; preterms: -24 CC 1.00 ± 0.26 (*n = 17*) and -24 CT 0.54 ± 0.24 (*n = 9*); Kruskal-Wallis-test: χ^2=0.384; df=1; p=0.535). Moreover, there was no effect of the -24C>T on the protein expression of MRP2 compared to the wild type (Mean relative band intensity ± SD; terms -24 CC 0.91 ± 0.08 *(n = 8)* and terms -24 CT 0.91 ± 0.19 *(n = 5)* Kruskal-Wallis-test: χ^2=0.05; df=1; p=0.826; preterms -24 CC 0.74 ± 0.08 *(n = 10)* and preterms -24 CT 0.52 ± 0.15 *(n = 6)* Kruskal-Wallis-test: χ^2=1.428; df=1; p=0.232). In summary, we did not find any influence of the genotype at position -24 in the 5`-untranslated region (5`-UTR) of the transporter gene on the expression of *MRP2* in human placenta.

Influence of the missense mutation of position 1249 on the expression of MRP2

In addition, the missense SNP 1249 G>A in exon 10 was investigated in relation to the MRP2 mRNA expression. The amount of mRNA in preterm placentas is significantly reduced in carriers of the variant allel (1249 AA and 1249 GA) in comparison to those carrying GG at position 1249 (MRP2 mRNA/18S rRNA-ratio x 1000 ± SD; preterms 1249 GG: 0.03 ± 0.02 *(n = 21*); preterms 1249 GA: 0.6 ± 0.28 *(n = 3);* preterms 1249 AA: 0.95 *(n = 2),* Kruskal-Wallis-test: χ^2=4.08; df=2; p=0.044). In term placentas there was no statistical significant difference in expression of the MRP2 mRNA in relation to genotype (MRP2 mRNA/18S rRNA-ratio x 1000 ± SD; terms 1249 GG: 1.62 ± 0.26 *(n = 16);* terms 1249 GA: 2.5 ± 0.39 *(n = 13);* terms 1249 AA: 2.92 ± 1.30 *(n = 3)*; Kruskal-Wallis-test: χ^2=3.768; df=2; p=0.152).

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Protein expression of MRP2 which has been established in a subset of the placental samples did not differ in relation to the G1249A gentotype (Mean relative band intensity ± SD; terms 1249 GG 0.77 ± 0.28 *(n = 6)* and terms 1249 GA 1.00 ± 0.30 *(n = 7);* Kruskal-Wallis-test: χ^2=1.479; df=1; p=0.224). However, homozygote AA-carriers had a slightly lower protein amount in preterm placentas (Mean related band intensity ± SD; preterms: 1249 GG 0.67 ± 0.24 *(n = 12)* vs. preterms 1249 GA 0.56 *(n = 2)* vs. preterms 1249 AA 0.47 *(n = 2);* Kruskal-Wallis-test: χ^2=1.895; df=2; p=0.428).

Influence of the C to T genotype in position 3972 on the expression of MRP2

Testing the influence of C3972T on the MRP2 mRNA level showed no difference in preterm (MRP2 mRNA/18S rRNA-ratio ± SD; preterms 3972 CC 0.90±0.37 *(n = 11)* vs. preterms 3975 CT 0.90 ± 0.25 *(n = 11)* vs. preterms 3972 TT 0.48 ± 0.39 *(n = 4);* Kruskal-Wallis-test: χ^2=0.690; df=2; p=0.708) or term placentas (MRP2 mRNA/18S rRNA-ratio ± SD; terms 3972 CC: 2.39 ± 0.52 *(n = 11)* vs. terms 3972 CT: 2.08±0.31 *(n = 16)* vs. terms 3972 TT: 1.63±0.52 *(n = 5);* Kruskal-Wallis-test: χ^2=0.828; df=2; p=0.661). Protein expression of MRP2 tended to be lower with carrying the variant genotype. In term placentas protein expression of MRP2 tend to be lower in 3972 TT carriers than in wild types (Mean relative band intensity ± SD; terms 3972 CC: 0.94 ± 0.1 *(n = 4)*; terms 3972 CT: 1.08 ± 0.10 *(n = 6)*; terms 3972 TT: 0.65 ± 0.08 *(n = 3)*, Kruskal-Wallis-test: χ^2=3.912, df=2, p=0.141), similar is the trend in preterm placentas (Mean band intensity ± SD; preterms 3972 CC: 0.74 ± 0,08 *(n = 7)*; preterms 3972 CT: 0.72 ± 0.19 *(n =5)*; preterms 3972 TT: 0.43 ± 0.14 *(n = 4)*, Kruskal-Wallis-test: χ^2=2.694, df=2, p=0.260).

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DISCUSSION:

In this study we show a significant increase of MRP2 mRNA and protein amounts (FIG. 1 and FIG. 2) as a function of gestational age. Knowledge about variation of the transporter expression is important for developing strategies to deliver drugs to the mother with minimal risk for the fetus or for delivering drugs to the fetus. The present paper demonstrates lower expression of MRP2 in preterm placentas. Moreover, the expression of MRP2 in the apical membrane of the multinuclear syncytiotrophoblasts of term placenta shown previously by St. Pierre et al. (St Pierre *et al.*, 2000) using immunohistochemistry was confirmed by additional methods as immunoblot analysis of separated apical and basal membrane fractions and by immunofluorescent microscopy staining (Fig.3 A and B).

Modulation of MRP2 expression is of high interest since this protein is involved in transport of physiological and pharmacological compounds and variable abundance may therefore be accompanied by a modulation of protective features of the placenta. Gestation implies an enrichment of the multinuclear syncytiotrophoblasts. This process is further actuated by subsequent maturation of cytotrophoblasts. Consequently, maturation of cytotrophoblasts is accompanied by higher expression of MRP2 as shown in cultured cytotrophoblasts (Fig 4 C).

Function and integrity of the multinuclear syncytium depends on fusion with cytotrophoblasts. It is assumed that expression of proteins is related to the capacity of absorbing cytotrophoblastic ribosomes and other structural proteins (Kingdom *et al.*, 2000). Therefore the primary induction of MRP2 mRNA on day two of culturing the progenitor cells could be related to the start of fusion. However, the following reduced amount of mRNA is not accompanied by a loss of protein as shown by Western blot analysis (FIG.5).

Elimination of fetally produced toxic compounds and protection of the fetus against maternal or exogenous substances are two major functions of the placenta. MRP2 which is expressed at the apical membrane of the syncytiotrophoblasts may contribute to both processes. In fact, MRP2 has been characterized to transport metabolites of phase II-biotransformation,

namely conjugates of lipophilic substances with sulphate, glucuronate and glutathione (Keppler *et al.*, 1996; Keppler *et al.*, 1997; Koenig J. *et al.*, 2003) including bilirubin glucuronides (Jedlitschky *et al.*, 1997) thereby playing a pivotal role in elimination of haemoglobin metabolites.

To date the *in vivo* regulation of MRP2 expression is poorly understood. There are different studies reporting an influence of xenobiotics on the expression level including cycloheximidine, 2-acetylaminofluorene and rifampin (Cole *et al.*, 1992; Kartenbeck *et al.*, 1996; Buchler *et al.*, 1996; Fromm *et al.*, 2000). Moreover, it has been reported that steroids in detail the glucocorticoid dexamethasone induces expression of the transporter. This effect is presumed to be mediated by PXR (Courtois *et al.*, 1999). Indeed, the pregnane X receptor (PXR), has been shown to regulate MRP2 in human liver (Kast *et al.*, 2002)

Besides different endogenous molecules are reported to be involved in the regulation of *MRP2* expression, for instance, a short term regulation of the MRP2 mediated efflux is associated with proteinkinase C (PKC) induction by the intracellular signalling molecule cGMP. It has been reported that induction of cGMP via endothelin-1 receptor B represses the expression of mrp2 in proximal tubule of killifish (Masereeuw *et al.*, 2000; Notenboom *et al.*, 2004). Another mechanism which has been reported to reduce the expression of MRP2 is the LPS-induced sepsis. It has been shown that inflammation due to an application of lipopolysaccharides (LPS) causes a decreased expression of drug metabolizing enzymes and eliminating transporters including mrp2 in rat intestine.

The change of plasma levels of hormones plays a major role in controlling the adaptation of maternal organism to the pregnancy. In detail, mean plasma levels of free and conjugated estrogens increase significantly with gestational age, reaching a peak few weeks before delivery. Estradiol is produced in large quantities from the feto-placental unit. Placental pregnenolon is metabolized to dehydroepiandrostenrone-3-sulphate (DHEAS) in the fetal adrenal glands, DHEAS returns to placenta. After uptake of DHEAS mediated by OATP-B and

OAT-4 (Ugele *et al.*, 2003) it is transformed to androstendione and then to estradiol in the syncytiotrophoblasts.

It is possible that parts of the active estradiol can be conjugated in the syncytiotrophoblasts as UDP-glucuronosyltransferase isoforms are expressed in these cells (Collier *et al.*, 2002a; Collier *et al.*, 2002b; Syme *et al.*, 2004). Metabolites of estradiol including estradiol-17 β-glucuronide and with a lower affinity estradiol-3 β-glucuronide are substrates of MRP2 (Gerk *et al.*, 2004). MRP2-mediated transport is assumed to be one of the main mechanisms of eliminating estradiol-17 β-glucuronide in liver shown by comparison of transport activities in rat liver canalicular membrane vesicle and *in vivo* elimination of intravenously administrated substance (Morikawa *et al.*, 2000). The changing expression of MRP2 in placenta as shown in this study can be associated with a higher transport activity of the placenta-mediated transport of cytotrophoblastic produced estradiol metabolites. The level of estradiol-17 β-glucuronide in peripheral maternal veins is higher than that of umbilical veins and arteries. However, it is assumed that this refers to a control of estradiol-17 β-glucuronide synthesis located in the maternal organism (Okada *et al.*, 1984).

The maternal mean plasma levels of estradiol-17 β-glucuronide increases during pregnancy. Gerk et al. postulate that the increase of this cholestatic metabolite of estrogens is responsible for the reduction of maternal bile efflux during normal pregnancies (Gerk *et al.* 2004). In fact, it has been reported that estradiol-17 β-glucuronide induces a partial internalization of Mrp2 during acute phase of cholestasis associated with a reduced bile flow. Pre-treatment with dibuturyl-cAMP reduces the endocytotic internalization of the transporter and the cholestatic effect (Mottino *et al.*, 2002). Recently we reported decreasing levels of MRP5 in human term placentas (Meyer zu Schwabedissen *et al.*, 2005). This transporter for cyclic nucleotides is assumed to be involved in controlling the intracellular level of signalling molecules by predominantly transporting cGMP, and with a lower affinity cAMP (Jedlitschky *et al.*, 2000). Assuming that cAMP is protective against estradiol-17 β-glucuronide induced

internalization it can be assumed that this is one of the mechanisms involved in the higher expression of MRP2 in term placentas.

Drug therapy of the unborn child is important in context of several diseases such as perinatal HIV infection. It has been shown that high active anti-retroviral therapy (HAART) is associated with the lowest rates of HIV transmission (Cooper *et al.*, 2002). HIV protease inhibitors like saquinavir, ritonavir and indinavir have been characterized as substrates for MRP2 (Gutmann *et al.*, 1999; Huisman *et al.*, 2002). High active antiretroviral therapy has been administered during the last weeks of pregnancy without achieving therapeutic drug concentrations in the fetal circulation (Forestier *et al.*, 2001; Marzolini *et al.*, 2002). This lack of transfer can be readily explained by the higher expression of MRP2 in later stages of pregnancy as indicated by our study. It is noteworthy, however, that based on animal experiments other ATP-transporters like MDR-1 and BCRP are associated with reduced drug accumulation in the fetal circulation (Jonker *et al.*, 2000; Pavek *et al.*, 2001).

The effect of various genetic polymorphisms of ABC transporters on drug disposition has been addressed in several studies. It has been shown for example, that the C3435T polymorphism in exon 26 of the *MDR1*-gene is associated with a lower P-glycoprotein mRNA-expression und efflux activity (Hitzl *et al.*, 2001). These results correlate with the steady-state concentrations of digoxin in Caucasian volunteers. Carriers of the 3435T showed higher AUC and C(max) values compared to those carrying the wild type gene (Hoffmeyer *et al.*, 2000; Johne *et al.*, 2002).

Various polymorphisms in the MRP2 gene contribute to the aetiology of the Dubin Johnson syndrome (Paulusma *et al.*, 1996; Wada *et al.*, 1998; Tsujii *et al.*, 1999; Mor-Cohen *et al.*, 2001; Tate *et al.*, 2002; Materna and Lage, 2003; Wakusawa *et al.*, 2003). It was shown that single nucleotide changes like the 3517 A/T transition in exon 25 are associated with lower efflux of MRP2-substrates and altered distribution of the protein in transfected cells (Mor-Cohen *et al.*, 2001; Keitel *et al.*, 2003). Impaired protein maturation followed by proteasomal

degradation of the transporter has been suggested as an explanation (Keitel *et al.*, 2003) Similar results have been shown for the C2302T missense mutation (exon 18) (Toh *et al.*, 1999; Hashimoto *et al.*, 2002). Furthermore, different polymorphisms of MRP2 with unknown functional consequences have been described (Ito *et al.*, 2001; Gerk and Vore, 2002)

In the present paper we studied the effect of three single nucleotide polymorphisms on the mRNA expression in human placenta namely the C-24T, G1249A and C3972T. The C-24T polymorphism in the 5`-untranslated region of MRP2 was not associated with significant changes in MRP2 mRNA or protein expression. These findings are in agreement with previously described results in duodenal samples of Japanese healthy subjects (Moriya *et al.*, 2002). Accordingly, characterization of the 5`-flanking region of the human *MRP2* gene showed no binding sequences for known vertebrate-encoded transcription factors in position -24 of the *MRP2* gene (Stockel *et al.*, 2000). Moreover, there was no statistically significant alteration of the MRP2 expression in term and preterm placentas as a function of the exon 28 polymorphism.

We found, however, a significant influence of the G1249A missense mutation on mRNA level in the present study. Our data suggest that 1249 AA is associated with a lower expression of MRP2 mRNA in human preterm placenta with also a trend in lower protein expression, however, there was no statistically significant difference observed in term placentas. In view of the low small sample number, however, confirmation in a larger population is mandatory. The exact mechanism for different expression levels of MRP2 as a function of non-synonymous genetic variants is unknown. A similar phenomenon has been described for MDR 1 and the following mechanisms have been proposed: altered translation efficiency or allele-specific differences in RNA folding (Eichelbaum *et al.*, 2004).

Taken together, our study indicates that MRP2 expression in human placenta is affected by gestational age with increased protein concentrations at later stages of pregnancy. Therefore, the mature fetus shows a better protection against xenobiotics, which are substrates for MRP2.

Likewise cellular differentiation *in vitro* was accompanied by increased expression of MRP2. This phenomenon can be also of clinical relevance in the setting of drug treatment during pregnancy.

ACKNOWLEDGMENTS:

We thank M. Grube, K. May, N. Siebert, S. Kuno and T. Brüggmann for technical and methodical support. Moreover, we thank Prof. Straube (Division of Gynecology) for the good collaboration.

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Footnotes:

Financial support: Karl & Lore Klein Stiftung, Oy-Mittelberg, Germany

DMD Fast Forward. Published on April 8, 2005 as DOI: 10.1124/dmd.104.003335
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Figure Legends:

Figure 1: Expression of *MRP2* as a function of gestational age. MRP2 mRNA amount was determined by Real-time PCR and normalized to 18S rRNA amount. Preterm placentas of a gestational age lower than 32 weeks of pregnancy (*left column*) show a significantly (*1 $p < 0.05$, Student´s t-test) lower expression of the transport protein than preterm placentas of a gestational age between 32 and 37 weeks (*middle column*). Moreover, there is a statistical significant increase of the mean *MRP2* mRNA-expression in term placentas (*right column*) compared to early preterm placentas or late preterm placentas (*1 $p < 0.05$ and *3 $p< 0.001$, respectively). Data are expressed as MRP2 mRNA/18S rRNA-ratio (x 1000) (mean values ± SD; early preterm placentas *n= 10*; late preterm placentas, *n= 16*; term placentas, *n=32*).

Figure 2: Immunoblot analysis of the MRP2 amount in placental samples. Crude membrane fractions were separated by SDS-PAGE and transferred to nitrocellulose filter. Detection was performed using an anti- MRP2-antibody M2 I-4 (Alexis Biochemicals, Gruneberg Germany) at a dilution of 1:1000. Figure 2A shows staining of protein extract of early preterm placentas and Figure 2B that of term placentas. Moreover Figure 2C shows staining of preterm and term placentas. Band intensity was analyzed using KODAC Image Software and normalized to the internal standard (IS) consisting of a crude membrane preparation of a term placenta. Human term placentas show a significantly (* $p< 0.05$, Student‘s t-test) higher expression of MRP2 than preterm placentas (Fig. 2D). Data are expressed as mean relative band intensity ± SD. As positive control human liver (HL) was used.

Figure 3: Detection of the localization of MRP2 in human placenta. Figure 3A shows an immunoblot analysis of MRP2 in basal and apical membrane preparations of human term (T) and late preterm (P) placentas. Human liver was used as positive control. MRP2 is enriched in the fraction of apical membranes. Immunohistochemical staining of paraffin sections of placenta shows the localization in the syncytiotrophoblasts. Term placentas show an intensive staining whereas the immunofluorescent signal (Fig. 3B) in human preterm placentas appears to be less intensive (Fig.3C). Control staining with the secondary anti-mouse antibody was performed in term (Fig.3D) and preterm (Fig.3E) placenta.

Figure 4: Purity of isolated and cultured cytotrophoblasts was controlled by staining of maturated cells with an anti-cytokeratine 18 antibody (Fig. 4A). Moreover the cells were stained with an anti-vimentin antibody (Fig. 4B). Differentiation of the progenitor cells was established by light microscopy after 12h (Fig. 4C) and 144h (Fig. 4D) in culture, showing cell fusion and formation of a multinuclear syncytium (Fig. 4D). Maturation of cytotrophoblasts in culture was controlled by measuring the amount of β-human choriogonadotropin (β-hCG) in the supernatant data are expressed as mean value ± SD from four different isolations. As shown in Figure 4E the level of this pregnancy hormone, a biochemical marker of the activity of syncytiotrophoblasts, increases with differentiation of these cells in vitro.

Figure 5: Detection of the *MRP2* in cultured cytotrophoblasts. Analysis of the amount of MRP2 mRNA normalized to 18S rRNA shows an increase of the transporter within the first two days of culture followed by a reduction (mean MRP2 mRNA/18S rRNA- ratio x 1000 ± SD; n= 4 different isolations). In addition, the amount of MRP2 protein was

determined by Western Blot analysis. Comparing the expression at day 3 and 6 with that of cytotrophoblast shows that the maturation of these progenitor cells is accompanied by increasing levels of MRP2 expressed in the membrane (insert).

Figure 6: Genotype of position 1249 which is located to exon 10 of the *MRP2* gene related to the normalized MRP2 mRNA-amount in human preterm placentas. The amount of *MRP2* mRNA is decreased in preterm placentas carrying the mutant allel (A) in position 1249 compared to those harbouring the homozygous wildtype genotype (1249 GG). Data are expressed as mean MRP2 mRNA/18S rRNA-ratio ± SD; * $p<0.05$ Kruskal-Wallis-test).

TABLES:

Table 1: Demographic data

	Term Placentas	**Preterm Placentas**	
	> 37 weeks of gestation	32-37 weeks of gestation	23-32 weeks of gestation
Number	32	16	10
Mean age of the pregnant	29.0±5.8	28.5±6.3	27.2±6.5
Mean weeks of gestation	39+3 days (*)	34+4 days (*)	28+3 days (*)
Number of gravidities	2.04 ± 0.94	2.13 ± 1.46	2.46± 1.89
Number of birth before	1.81 ± 0.84	1.71 ± 0.73	2.07± 1.75
BMI (at the beginning of gravidity)	24.11 ± 5.82	23.65 ± 3.74	21.88 ± 4.00
BMI (at the end of gravidity)	29.14 ± 5.68 (*)	27.75 ± 3.22 (*)	24.34 ± 3.89 (*)
Cesarian Sections	63%	73%	78%

BMI: body mass index, data are expressed as mean ± SD; (*) $p < 0.05$

Table 2: Primers used for amplification of fragments of the *MRP2*-gene containing previously described single nucleotide polymorphisms (SNPs)

Exon	Primer	Fragment length
Promotor	5´-TAAATGGTTGGATGAAAGG-3´	301 bp
	5´-GCTTTAGACCAATTGCACATC-3´	
Exon 10	5´-AACTTGGCCAGGAAGGAGTACAC-3´	260 bp
	5´-CTGGGTGACTTTTTCTTTACCTGAATG-3´	
Exon 28	5´-AACTTACTTCTCATCTTGTCTCCCTTGC-3´	184 bp
	5´-CTCCACCTACCTTCTCCATGCTAG-3´	

DMD Fast Forward. Published on April 8, 2005 as DOI: 10.1124/dmd.104.003335
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Table 3: C-24T G1249 ($Val_{417}Ile$) and C3972T variants of the *MRP2* gene in the population and in terms and preterms (preterm data in parenthesis)

Genotype	Number	Position	Identified frequency	Expected frequency	Allel	Allelic frequency
C/C	37	-24	63.8%	67.2%	C	0.82
	20 (17)		62.5% (65.4%)	65.6% (68.9%)		0.81 (0.83)
C/T	21	-24	36.2%	29.5%	T	0.18
	12 (9)		37.5% (34.6%)	30.8% (28.2%)		0.19 (0.17)
T/T	0	-24	0%	3.2%		
	0		0% (0%)	3.6% (2.9%)		
G/G	37	1249	63.7%	60.8%	G	0.78
	16 (21)		50.0% (80.8%)	49.0% (74.0%)		0.70 (0.86)
G/A	16	1249	29.3%	34.3%	A	0.22
	13 (3)		40.6% (11.5%)	42.0% (22.4%)		0.30 (0.13)
A/A	5	1240	8.6%	4.8%		
	3 (2)		9.4% (7.7%)	9.0% (1.8%)		
C/C	22	3972	39.7%	37.4%	C	0.62
	11 (11)		34.4% (46.1%)	35.2% (40.3%)		0.59 (0.65)
C/T	27	3972	46.6%	47.6%	T	0.38
	16 (11)		50% (42.3%)	48.4% (45.5%)		0.41 (0.35)
T/T	9	3972	15.5%	15.0%		
	5 (4)		15.6% (15.4%)	16.8% (12.2%)		

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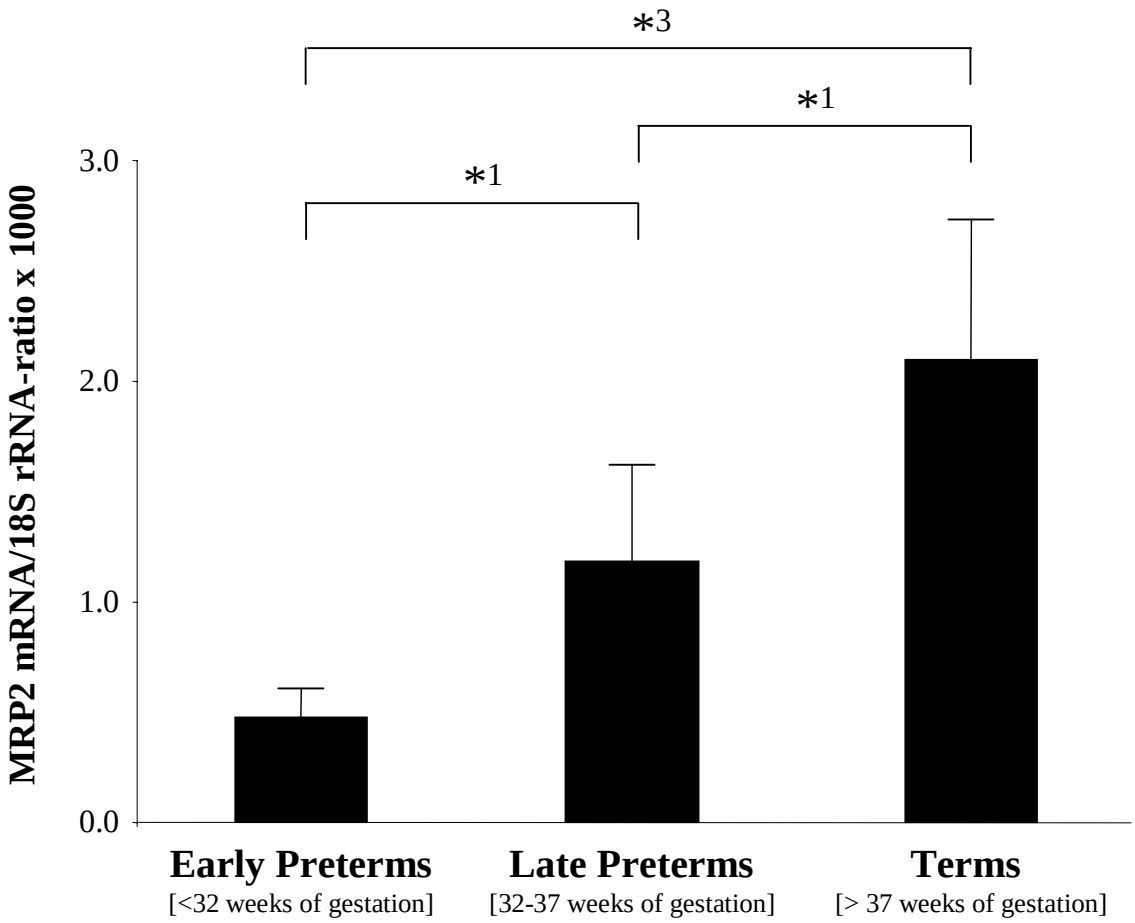

Figure 1

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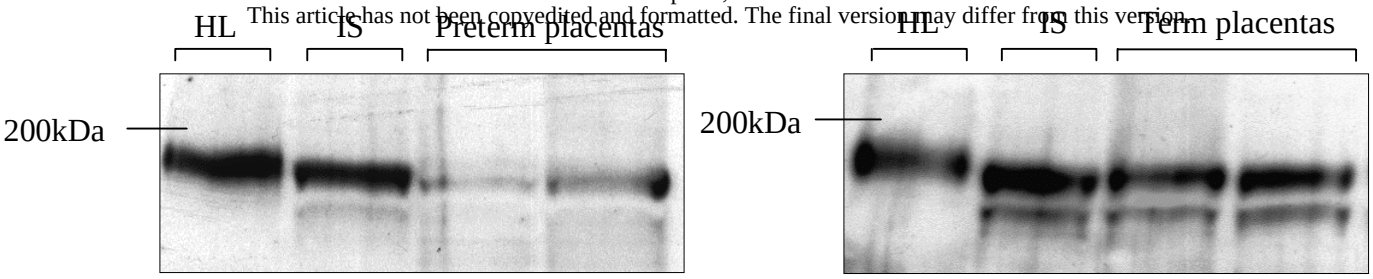
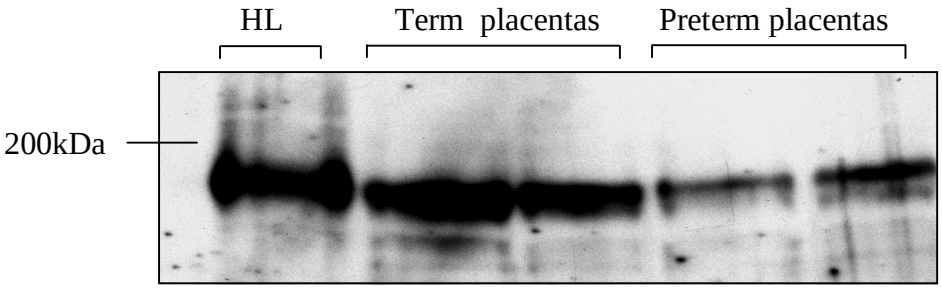


2A 2B



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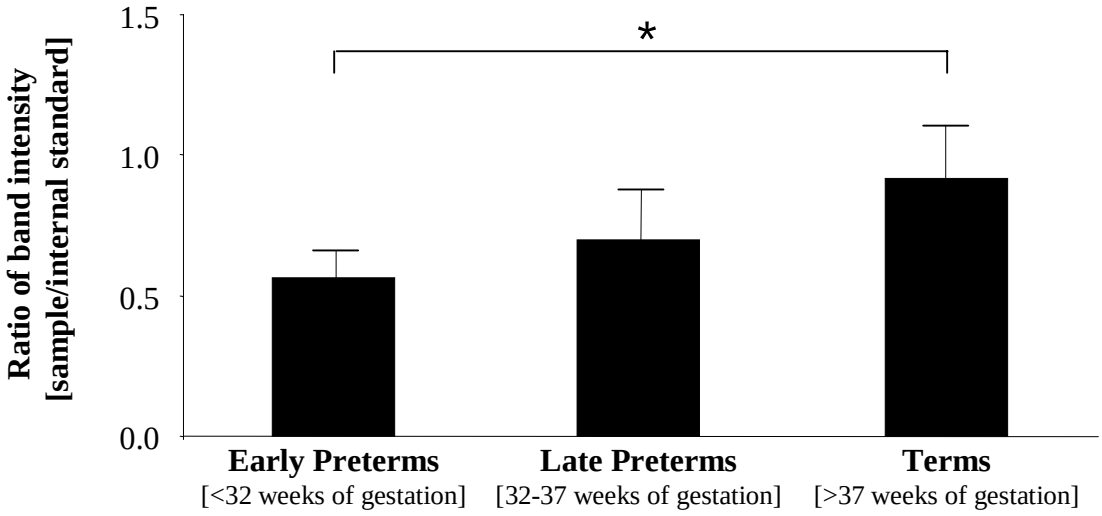

2D

Figure 2

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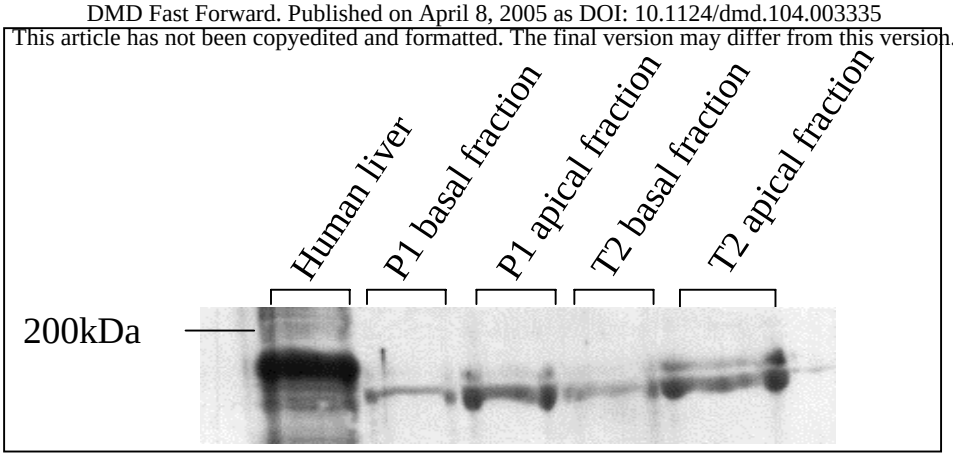
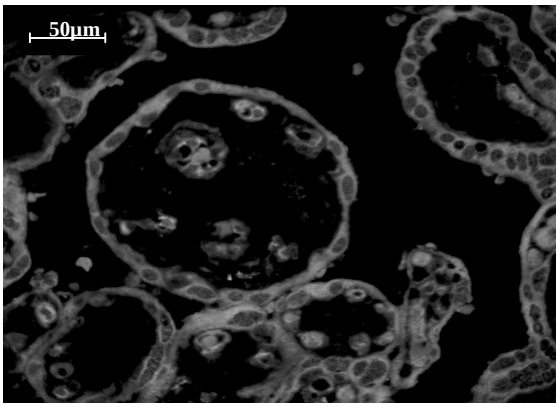


A



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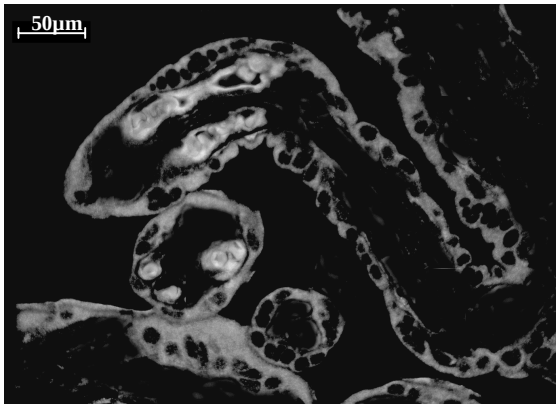

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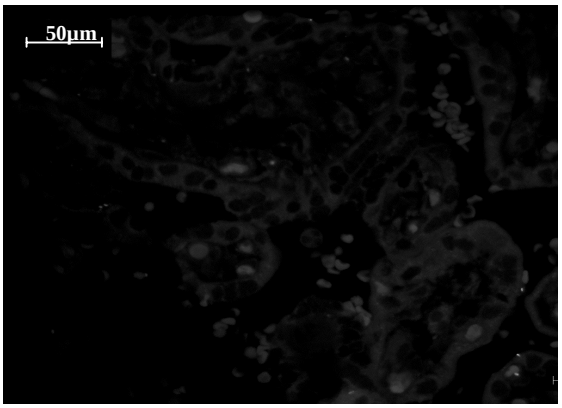

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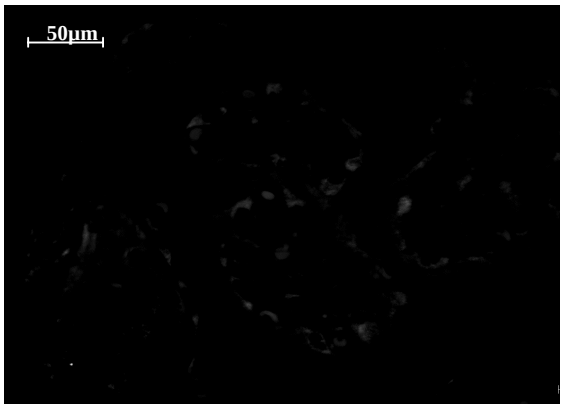

E

Figure 3

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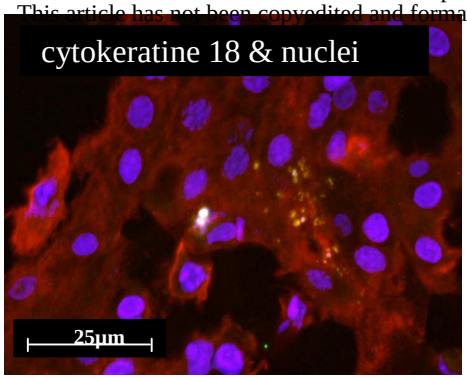
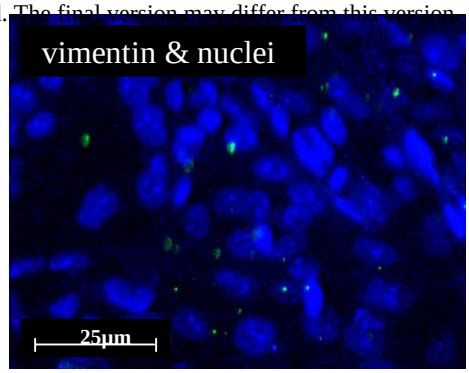


4A



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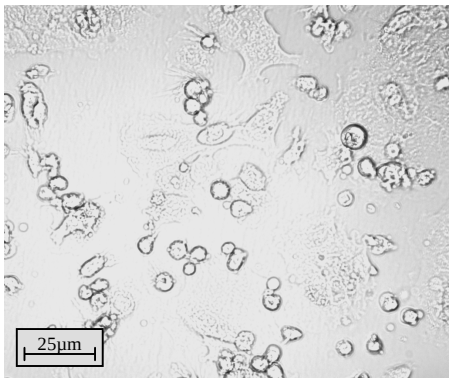

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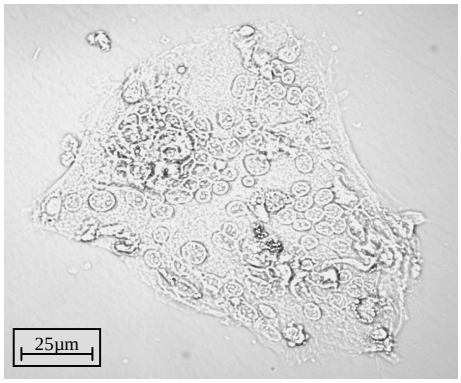

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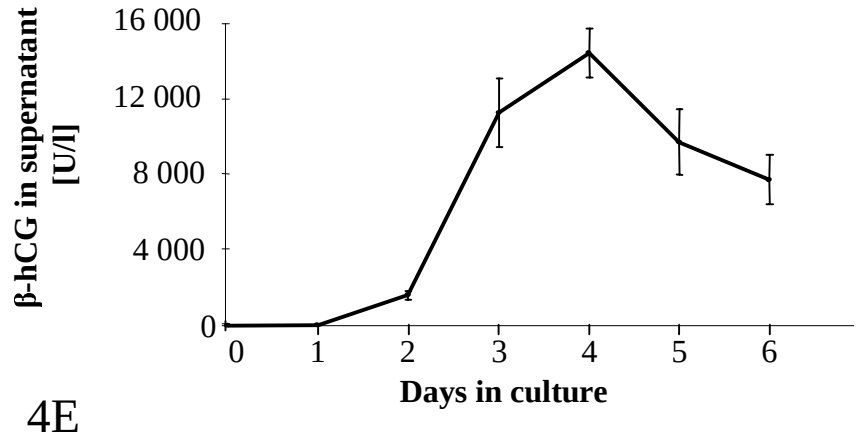

4E

Figure 4

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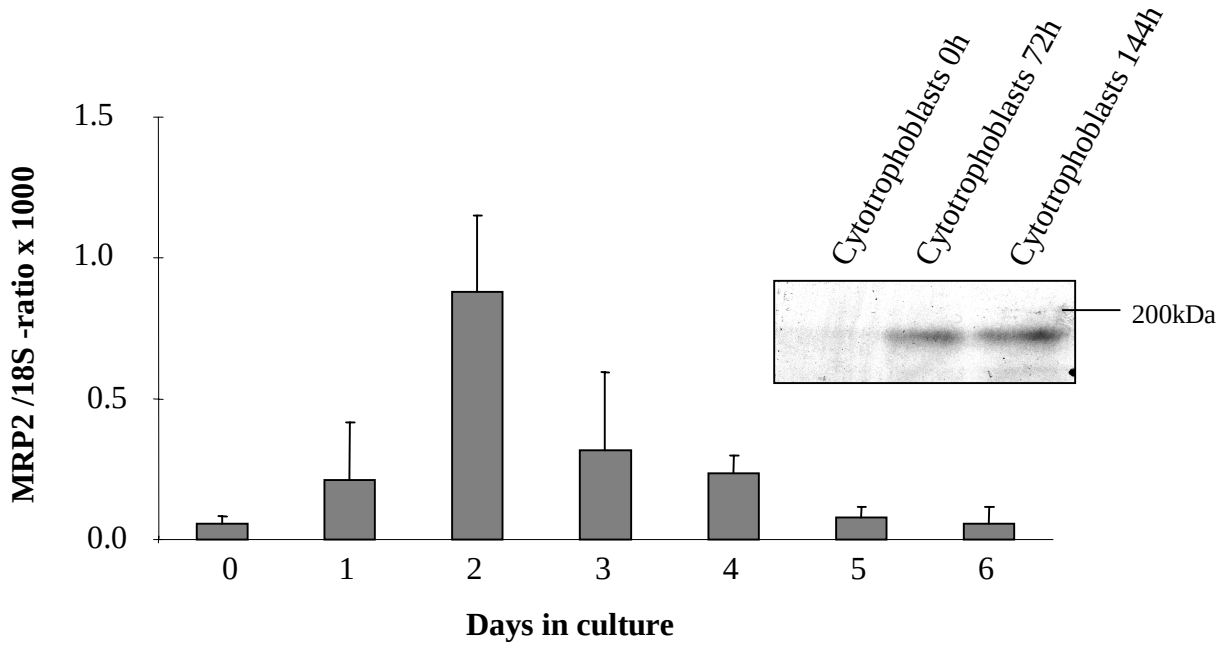

Figure 5

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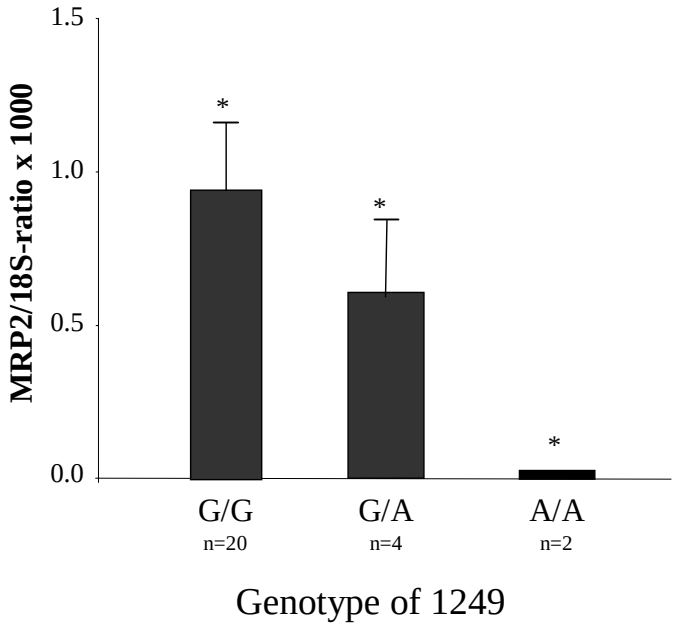

Figure 6